Prevalidation and Validation Study Plan for Minced Testes Assay

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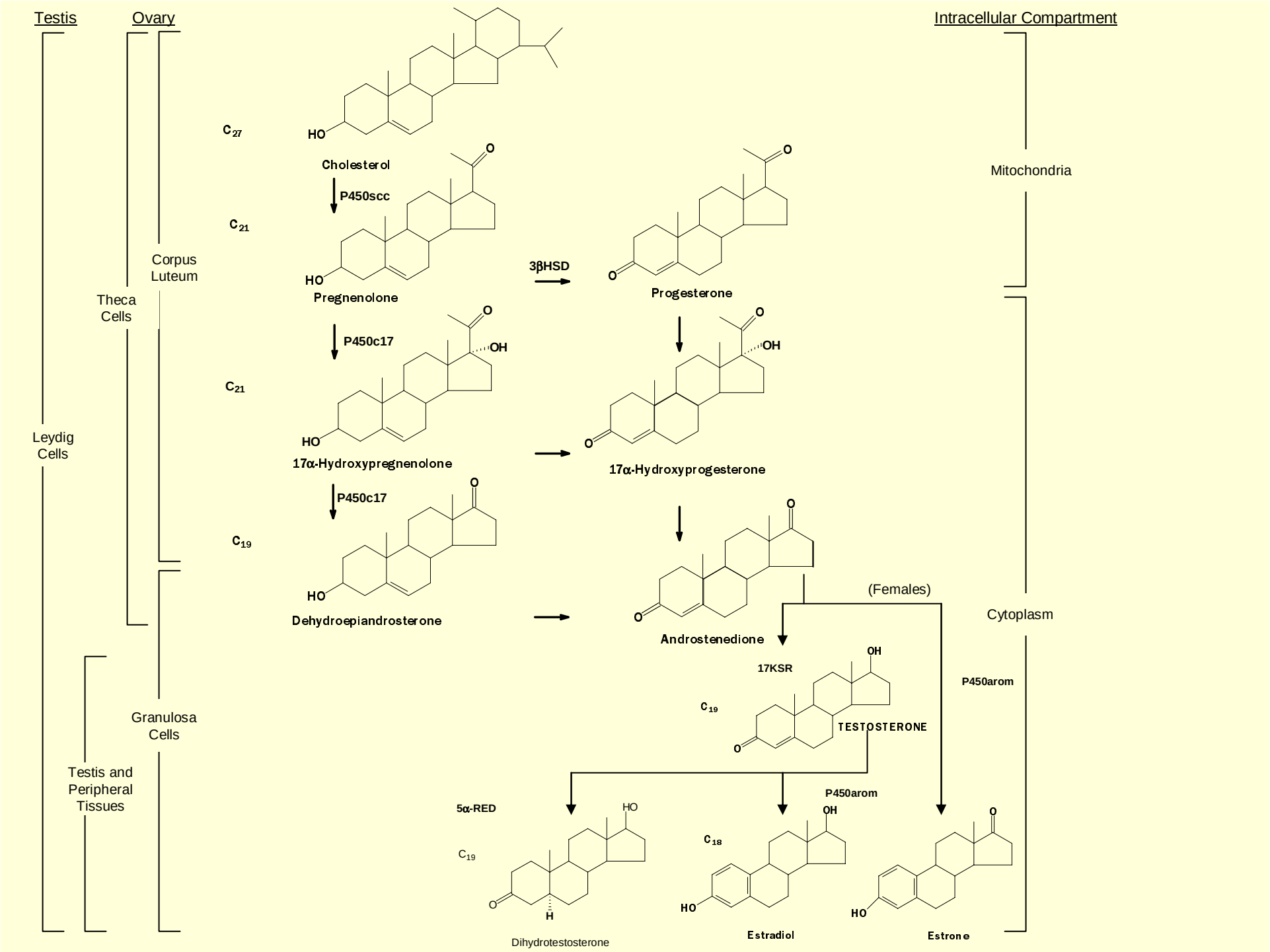
Objectives

- To assess relevance of minced testes assay for detecting compounds that affect steroidogenesis
  - Measure change in testosterone production relative to controls
- To assess reliability
  - Measure variability in testosterone measurements of participating laboratories
  - Measure variability in mean response among participating laboratories

Data Interpretation

- Assay will detect interference with key steps in the steroidogenic pathway:
  - Decreases in steroidogenic signal transduction
  - Interference with the transport of cholesterol from the cytoplasm to the mitochondria via StAR
  - Inhibition of enzymes involved in the conversion of cholesterol to testosterone


Testis
Ovary
Intracellular Compartment
Mitochondria
Cytoplasm
Leydig Cells
Theca Cells
Corpus Luteum
Granulosa Cells
Testis and Peripheral Tissues
C27
Cholesterol
P450scc
C21
Pregnenolone
3βHSD
Progesterone
P450c17
C21
17α-Hydroxypregnenolone
17α-Hydroxyprogesterone
P450c17
C19
Dehydroepiandrosterone
Androstenedione
(Females)
17KSR
C19
TESTOSTERONE
P450arom
5α-RED
C19
Dihydrotestosterone
P450arom
C18
Estradiol
Estrone

Data Interpretation (2)

- Interference with steroidogenesis will result in a decrease or increase in measured testosterone relative to controls
- Assay is unlikely to detect
  - Inhibition of aromatase
  - 5α-reductase inhibitors

  Inhibition of these downstream steps would result in an increase in testosterone production

Basic Protocol

- Optimization study will determine parameters of protocol
- Replicate Runs
  - 3 with hCG stimulation
  - 3 without hCG stiulation
- Dosing
  - 3 dose levels
  - 1 positive control
  - Media control
- Sample 3-4 time points

Prevalidation Studies

- Purpose:
  - To obtain initial information on protocol transferability
  - Primary test of relevance
- Studies:
  - Protocol optimization study
  - Baseline study
  - Pilot study
  - Multichemical study

Prevalidation Studies

- Optimization of minced testes protocol has been completed
- Baseline study
  - Two labs to run optimized protocol
  - 3 runs without hCG
  - 3 runs with hCG challenge
  - Measure testosterone formation and LDH
  - No test chemical
  - 3 replicates

Prevalidation Studies (2)

- Pilot studies
  - Aminoglutethimide (positive control)
  - Ethane dimethanesulfonate (Leydig cell toxicant)
  - Two labs
  - Three replicates
- Multichemical studies
  - 9 challenge chemicals
  - Two labs
  - Two replicates

Selection of Reference Chemicals

- Selected for known mode of action
- Limited by availability
  - Pharmaceuticals are difficult at best to procure because the require material transfer agreements
  - Many pharmaceuticals are not available from the manufacturer
- Thus, we will have to duplicate many of the chemicals in validation that were used in prevalidation, if we want to cover modes of action

Reference Chemicals

Chemical	Mode 1	Mode 2	Preval	Val
Aminogluteth imide	P450scc	Aromatase	+ Cont	+ Cont
Bisphenol A	Inhibits C-AMP		X	
Dimethoate	StAR inhibitor		X	X
EDS	Leydig cell toxicant		X	X
Fenarimol	Aromatase		X	

Reference Chemicals

Chemical	Mode 1	Mode 2	Preval	Val
Flutamide	P450c17		X	
Genistein	3β-HSD inhibitor		X	X
Ketoconazole	P450scc	Aromatase	X	
Lindane	Inhibits C-AMP			X
MK-434	5α reductase		?	
Vinclozolin	Negative chemical		X	X

Selection of Laboratories

- Laboratories to be selected by the Contractor by open solicitation
- Laboratories must be:
  - Independent
  - Experienced in:
    - In vitro test methods
    - Cell and tissue culturing
    - Test chemical administration
    - Enzyme kinetics and inhibition studies
  - Knowledge of steroidogenesis
  - Compliance with GLP

Measurements of Reliability

1. Coefficient of variation across studies
   - Study standard deviation/mean of studies
   - Reflects the spread among study means in relation to their average value
2. Ratio of between- to within-study standard deviation
   - Standard deviation across studies/average standard error within studies
   - Reflects relative contribution to total variation of the variability among study means as compared to the precision within studies
3. Comparison of within-lab SD to Average within-lab SD
   - Standard deviation of lab I/ geometric mean within-lab SD
   - Measures the homogeneity of within study variation across laboratories
   - Can identify poor performing labs

Determination of Number of Laboratories

- Sensitivity analysis for each measure of reliability was prepared using literature values. (Fail, Gray Laskey)
- For Criterion 1 (CV interval factor):
  - 95% confidence interval factors were calculated as a function of the number of laboratories and the number of replicate determinations per laboratory.
  - A 95% confidence interval on the characteristic of interest is calculated by multiplying the point estimate by the confidence interval factor.
  - Confidence interval factor is sensitive to the number of labs and approaches 1 as the number of labs increases, flattens after ~8 labs
  - Criterion 1 is not sensitive to the number of replicate determinations per lab

Determination of Number of Laboratories (2)

- Criterion 2 (Lower and Upper confidence interval factor of between:within SD)
  - 95% confidence interval factors were calculated as a function of the number of laboratories and the number of replicate determinations per laboratory
  - Lower confidence interval is sensitive to both number of labs and number of replicates
  - Upper confidence level is sensitive only to the number of labs
  - Flattens out after ~8 labs

Determination of Number of Laboratories (3)

- Criterion 3 (Lower confidence factor of within-laboratory standard deviations to average within-laboratory standard deviation)
  - 95% confidence interval factors were calculated as a function of the number of laboratories and the number of replicate determinations per laboratory
  - Sensitive to number of replicates
  - Not sensitive to number of labs
  - Flattens after ~8 replicates

Determination of Number of Laboratories (4)

- Conclusions
  - Based on available data, 6-10 laboratories are needed to achieve a high confidence indication of assay reliability
  - We shall select 6 laboratories as the actual variability in these studies should be less than in the literature where different protocols were used
  - ~8 replicates are needed to obtain a high confidence estimate of within laboratory standard deviations. This information will be generated by the positive and negative controls.

Validation

- **6 labs**
- **Baseline studies**
  - 3 runs without hCG
  - 3 runs with hCG challenge
  - Measure testosterone formation and LDH
  - No test chemical
- **Pilot studies**
  - Aminoglutethimide (positive control)
  - Ethane dimethanesulfonate (Leydig cell toxicant)

Validation

- **Coded sample studies**
  - 5 chemicals
  - 2 replicates per laboratory
  - Aminogluthethimide is positive control
  - Vinclozolin (AR antagonist) used as negative chemical
- Will modify validation study plan based on results of prevalidation work

Reference Chemicals by Mode of Action

Mode of Action	Prevalidation	Validation
C-AMP inhibitor	BPA	Lindane
StAR inhibitor	Dimethoate	Dimethoate
P450scc	+ Cont Ketoconazole	+ Cont
P450c17	Flutamide	
3β-HSD	Genestein	Genestein
5α reductase	MK-434	
Aromatase	Fenarimol	
Negative	Vinclozolin	Vinclozolin

Data Analysis

- Intra-Laboratory Analysis

  Assess chemically related testosterone inhibition within laboratories

- Inter-Laboratory Analysis

  Assess extent of heterogeneity of chemical inhibition effects across laboratories

Data Analysis Strategy

- Large numbers of comparisons can be identified, for both intra and inter laboratory analyses
  - Baseline studies
    - Preval: 2 labs x 3 replicates = 6
    - Validation: 6 labs x 2 replicates = 12
  - Positive Control (aminoglutethimide)
    - Preval pilot: 2 labs x 3 replicates = 6
    - Validation pilot: 6 labs x 2 replicates = 12
    - Preval high dose: 2 labs x 9 chems x 2 replicates = 36
    - Validation high dose: 6 labs x 5 chems x 2 reps = 60

Data Analysis Strategy (2)

- **Preval/val chemicals**
  2 labs x 4 chems x 2 replicates = 16
  6 labs x 4 chems x 2 replicates = 48

- **Preval chemicals**: 2 labs x 5 chems x 2 replicates = 20
- **Validation chemicals:** 6 labs x 1 chem x 2 replicates = 12

Total of 228 studies

- In validation study carry out each analysis considered *a priori* to be possibly toxicologically relevant
- Make recommendations in final report concerning which analyses are most informative and so should be included in assay standard practice

Intra-Laboratory Analysis

- Principal Endpoints
  - Cumulative Testosterone Concentration
  - Cumulative LDH Concentration

  (3 doses and 3-4 time points)

- Similar analyses for both endpoints

- Variation in Effects
  - Across chemicals
  - Across graded chemical doses

Components of Variation

- Rat-to-rat
- Testis-to-testis within rat
- Fragment-to-fragment within testis
- Block of assays performed simultaneously (e.g. day-to-day)

- Variance components need to be accounted for in the statistical analysis

Inter-Laboratory Analysis

- Focus on primary and secondary responses from intra-laboratory analysis
- Assess extent of heterogeneity of responses across laboratories
- Reference
  - American Society for Testing and Materials (1988). "Standard Practice for Conducting an Inter-Laboratory Study to Determine the Precision of a Test Method"

Measures of Variation Among Laboratories

- Heterogeneity of within-laboratory means across laboratories
- Heterogeneity of within-laboratory standard deviations across laboratories
- Ratio of laboratory-to-laboratory standard deviation to average within-laboratory standard deviation
- Coefficient of variation across laboratories

Reporting

- Each laboratory will report:
  - That protocol was followed
  - Difficulties in executing the studies
  - Summary of data
  - Raw data
- Validation Study Report

Graphical Summary Displays

- Prepare control charts and associated control limits
- Display intra-laboratory statistics side-by-side across
  - Chemicals
  - Graded doses
  - Laboratories
- Identify outlying laboratories and nature of discrepancies